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The "Phosphoryl-Enzyme" from Phosphoglycerate Kinase†

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Appendix: Crystalline 3-Phospho-D-glycerate Kinase from Horse Muscle†

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ABSTRACT: The "phosphoryl-enzyme" prepared from phosphoglycerate kinase and adenosine 5′-triphosphate in the presence of an adenosine 5′-diphosphate trap is shown to contain stoichiometric amounts of 3-phosphoglycerate. This "phosphoryl-enzyme" is chemically competent, but is probably just a tight complex between 1,3-bisphosphoglycerate and the enzyme. The two partial exchange reactions (between adenosine 5′-diphosphate and adenosine 5′-triphosphate, and between 3-phosphoglycerate and 1,3-bisphosphoglycerate) can both be observed, but their rates are very much slower than the rate of overall catalysis. No substrate analogue was found that accelerated the partial exchange reactions. Catalysis of each of the two exchange reactions and of the kinase reaction coincides after isoelectric focusing of purified enzyme, but the amount of cosubstrate necessary to cause the observed partial exchange rates is so small that these reactions may well be artifactual. The balance of evidence does not support a ping-pong pathway *via* phosphoryl-enzyme, and the reaction may be a sequential one in which the phosphoryl group is transferred between substrates in a ternary complex. The results point to the dangers in the interpretation of experiments where very small amounts of contaminating cosubstrate can lead to large kinetic effects, and to the possibility of mistaken deductions about the identity of reaction intermediates.

Phosphoglycerate kinase (ATP-3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3), which catalyzes the transfer of a phosphoryl group between 1,3-bisphosphoglycerate and ADP, has been the subject of considerable mechanistic controversy. The elucidation of the mechanism is complicated by conflicting evidence from experiments using several different techniques. Steady-state kinetic data (Larsson-Raźniekiewicz, 1967; Larsson-Raźniekiewicz and Arvidsson, 1971; Janson and Cleland, 1974; Lee and O'Sullivan, 1975) indicate that the mechanism may be of the "random sequential" type. Evidence suggesting that the phosphoglycerate kinase catalyzed reaction proceeds *via* a pathway that involves a discrete, isolable, covalent phosphoryl-enzyme intermediate (Walsh and Spector, 1971; Roustan et al., 1973; Brevet et al., 1973) has also been reported. The chemical competence of the postulated phosphoryl-enzyme was demonstrated by the complete transfer of phosphate to ADP and to 3-phosphoglycerate (3-PGA)[1] (Walsh and Spector, 1971). The "U"-shaped pH–stability profile of the phosphoryl-enzyme and the observation that hydroxylaminolysis led to the facile release of phosphate (Walsh and Spector, 1971) suggested the presence of an acyl phosphate linkage. Indeed, Brevet et al. (1973) have proposed that the phosphoryl group is attached to the γ-carboxyl group of a glutamyl residue, on the basis of experiments in which the isolated phosphoryl-enzyme was treated with hydroxylamine and the product subjected to a Lossen rearrangement. Further evidence for the existence of a phosphoryl-enzyme has come from the reported partial isotopic exchange reaction between ATP and [^{3}H]ADP, apparently in the absence of the cosubstrate (Walsh and Spector, 1971; Roustan et al., 1973). The concept of "substrate synergism" (Bridger et al., 1968) was invoked, since this exchange reaction was markedly accelerated by low concentrations of 3-PGA.

Certain inconsistencies, however, caused us to reinvestigate the nature of this apparent phosphoryl-enzyme. For instance, the rate of the partial isotopic exchange reaction is very slow, the proportion of yeast enzyme that can be phosphorylated is capriciously variable, and the horse enzyme cannot be phosphorylated. Moreover, if the mechanism does involve a viable

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[1] Abbreviations used are: 3-PGA, 3-phospho-D-glycerate; BPGA, 1,3-bisphospho-D-glycerate; E ~ P, phosphoryl-enzyme; P_i, inorganic phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

phosphoryl-enzyme, then a partial exchange reaction between 1,3-bisphosphoglycerate (BPGA) and [^{14}C]3-PGA should exist in the absence of ADP, and the production of the phosphoryl-enzyme from BPGA should also be possible (see Scheme I). A preliminary report of some of this work has appeared (Johnson et al., 1975).

Experimental Procedure

Materials

Phosphoglycerate kinase was isolated from yeast (Scopes, 1971) and from horse muscle (see Appendix). The yeast enzyme was also purchased from Boehringer Corp. and from Sigma. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was obtained from Boehringer. Rabbit muscle adenylate kinase, pyruvate kinase, lactate dehydrogenase, phosphoglycerate mutase, and yeast enolase were obtained from Sigma. [^{32}P]Orthophosphate, [2-^{3}H]ADP, and [2-^{3}H]ATP were obtained from the Radiochemical Centre. [γ^{32}P]ATP was prepared by the method of Glynn and Chappell (1964). 3-Phospho-D-[1-^{14}C]glyceric acid was prepared by the method of Sjödin and Vestermark (1973). [^{14}C]Glyceraldehyde 3-phosphate was prepared from 3-phospho-D-[1-^{14}C]glycerate using the method of the assay for 3-PGA (Orr and Knowles, 1974) omitting the hydrazine.

ADP, ATP, and NADH were obtained as their sodium salts from Boehringer. 3-Phospho-D-glyceric acid (tricyclohexylammonium and barium salts), 3-phospho-DL-glyceraldehyde diethyl acetal (barium salt), dihydroxyacetone phosphate dimethyl ketal (bis(monocyclohexylammonium) salt), phospho-D-serine, phospho-L-serine, DL-α-glycerophosphate (disodium salt), phosphoglycolic acid (tris(monocyclohexylammonium) salt), phosphohydroxypyruvic acid, and β-NAD$^+$ (free acid) were obtained from Sigma.

All buffers were prepared from analytical grade reagents using deionized distilled water.

3-Phospho-L-glyceric Acid. This compound was prepared by the deamination of phospho-L-serine with nitrous acid. The product had ir and NMR spectra identical with those of authentic 3-phospho-D-glyceric acid. The L isomer was contaminated by ca. 1% of D isomer. This was removed enzymatically by conversion into pyruvate. The incubation mixture (3 ml) contained 50 mM triethanolamine-HCl, pH 7.1, KCl (50 mM), $MgCl_2$ (20 mM), ADP (15 mM), L-3-PGA (83 mM), D-3-PGA (0.76 mM), phosphoglycerate mutase (30 units), enolase (15 units), and pyruvate kinase (80 units). After incubation at room temperature for 15 h the mixture no longer contained any detectable D isomer. The L-3-PGA was diluted with distilled water (150 ml) and then chromatographed on a DEAE-52 column (1.5 × 17 cm) eluting with 5–300 mM ammonium bicarbonate (pH 8.0). The L-3-PGA (which was located using high-pressure liquid chromatography on AX-corasil in 30 mM phosphate buffer, pH 6.5) was found to cochromatograph with AMP. The appropriate fractions were pooled, buffer was removed in vacuo at 35 °C, and the residue was dissolved in water (40 ml). L-3-PGA and AMP were separated on a column (0.9 × 17 cm) of Dowex-1 (Cl^-) by elution with a linear gradient of HCl (0–200 mM).

D-Glyceric Acid 3-Sulfate. This was prepared as described by Fitzgerald et al. (1971).

Phosphoglycolohydroxamic Acid. This was prepared according to the method of Collins (1974).

3-Phospho-D-glycerylhydroxamic Acid. This compound was prepared either enzymatically from 3-PGA using phosphoglycerate kinase and hydroxylamine or chemically by the hydroxylaminolysis of the 3-phosphomethyl-D-glycerate (prepared by Fischer–Spier esterification of 3-phosphoglyceric acid). For the enzymatic synthesis, the incubation mixture (3 ml) contained: ATP (37 mM), 3-PGA (35 mM), hydroxylamine sulfate (183 mM), EDTA (0.17 mM), triethanolamine–HCl (pH 7.4) (5 mM), and yeast phosphoglycerate kinase (200 units). After incubation at room temperature for 15 h, an hydroxamate assay (Krimsky, 1959) indicated that the reaction was complete. The mixture was chromatographed on a DEAE-52 column (20 × 1.5 cm) eluting with a gradient of ammonium bicarbonate (5–300 mM). The fractions containing hydroxamate were located using ferric chloride. Buffer was removed from the pooled fractions in vacuo at 30 °C. The yield of the hydroxamate was 40–50 μmol.

The purity of the hydroxamate was checked by TLC on cellulose MN-300 plates in two different systems: acetone–acetonitrile–1 N HCl (64:26:10) and methanol–pyridine–H_2O–NH_3 (60:30:30:15). Hydroxamic acid and phosphate-containing compounds were detected using ferric chloride and Hanes–Isherwood sprays, respectively.

Methods

All ultraviolet absorption measurements were made on a Unicam SP 1800 spectrophotometer. pH measurements were made with a Radiometer TTT1C pH meter fitted with a pHA 630 scale expander. Scintillation counting was carried out on an automatic Beckmann Model LS 100 instrument. High-pressure liquid chromatography was carried out on a Waters Model ALC-202/R-401 liquid chromatograph with uv and RI detectors.

Phosphoglycerate kinase was assayed spectrophotometrically by the method described in the Appendix. The concentrations of kinase solutions were determined by using $E_{280}^{0.1\%}$ 0.49 for the yeast enzyme (Krietsch and Bücher, 1970) and 0.69 for the horse enzyme (see Appendix). A molecular weight of 45 000 was assumed for the yeast enzyme (Krietsch and Bücher, 1970) and 47 000 for the horse enzyme (see Appendix).

Adenylate kinase was assayed by the method of Chiu et al. (1967).

3-Phospho-D-glycerate was determined enzymically by the method of Orr and Knowles (1974).

ATP–ADP Exchange. This exchange was studied using a modification of the method of Walsh and Spector (1971). The incubation mixture (200 μl) contained: 200 mM triethanolamine-HCl, pH 7.8, $MgCl_2$ (8 mM), EDTA (0.5 mM), dithiothreitol (10 mM), ATP (3.0 mM), ADP (0.5 mM), and phosphoglycerate kinase (5–10 μg). Glycerol (10% v/v) was added to the incubation mixture in experiments with the horse enzyme. After incubation at 30 °C for 15 min, [^{3}H]ADP (500 mCi/mmol; 1 mCi/ml) (1 or 2 μl) was added to initiate the exchange reaction. At appropriate time intervals, samples (5 μl) were applied to a thin-layer plate (MN polygram cel 300 PEI) and developed in 0.52 M sodium phosphate buffer, pH 4.2 (R_f values: ATP, 0.15; ADP, 0.39). Nucleotide zones, which were located by their ultraviolet absorption, were cut out and counted in a scintillation counter. The isotopic exchange rate was calculated from a plot of log $(1 - A_t/A_\infty)$ where A = cpm in ATP/(cpm in ATP + cpm in ADP).

3-PGA–BPGA Exchange. This partial exchange reaction was studied by following the rate of incorporation of ^{14}C from 3-phospho[^{14}C]glycerate into glyceraldehyde 3-phosphate. The incubation mixture (170 μl) contained: 200 mM triethanolamine-HCl, pH 7.8, sodium citrate and $MgCl_2$ at concentrations (40 mM and 35 mM, respectively) to provide a

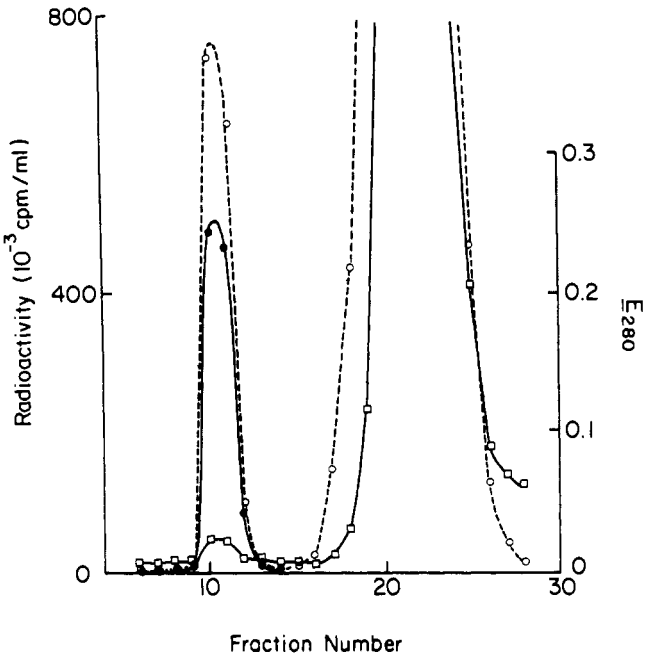


FIGURE 1: Phosphorylation of phosphoglycerate kinase by ATP. The incubation mixture (1.0 ml) contained: 50 mM triethanolamine-HCl, pH 7.5, $MgCl_2$ (4 mM), dithiothreitol (4 mM), [2-^{3}H]ATP (0.3 mM; 1.00×10^8 cpm/μmol), [γ^{32}P]ATP (0.3 mM; 4.34×10^8 cpm/μmol), phosphoenolpyruvate (0.1 mM), pyruvate kinase (40 units), and phosphoglycerate kinase (from yeast, 740 units, 20 μM). After incubation at room temperature for 60 min the reaction mixture was applied to a column (0.9 × 60 cm) of Sephadex G-25 (fine) equilibrated with 50 mM triethanolamine-HCl, pH 7.5. Fractions of 1.25 ml were collected at a flow rate of 12 ml/h and assayed for phosphoglycerate kinase activity. ^{3}H (□—□), ^{32}P (○- - -○), and E_{280} (●—●). Protein-containing fractions were subsequently boiled for 10 min, centrifuged to remove precipitated protein, and assayed for 3-phospho-D-glycerate by the method of Orr and Knowles (1974).

"magnesium buffer" with free [Mg^{2+}] near to 1 mM, glyceraldehyde 3-phosphate (4 mM), 3-PGA (1 mM), NAD^+ (1 mM), P_i (1 mM), glyceraldehyde-3-phosphate dehydrogenase (25 units), and phosphoglycerate kinase (10–40 units). After incubation at 30 °C for 15 min, [^{14}C]3-PGA (10 mM, 4 mCi/mmol) was added to initiate the exchange reaction. At appropriate time intervals, samples (10 μl) were injected onto a column of AX-corasil and eluted with 50 mM sodium phosphate buffer, pH 6.6, and the fractions containing glyceraldehyde 3-phosphate and 3-PGA were collected and counted. The isotope exchange rate was obtained from plots of log $(1 - R_t/R_e)$ where R_t = cpm in glyceraldehyde 3-phosphate at time t/total cpm and R_e = cpm in glyceraldehyde 3-phosphate at equilibrium/total cpm.

Isolation of E~P from BPGA. BPGA was prepared in situ from D-glyceraldehyde 3-phosphate and P_i using glyceraldehyde-3-phosphate dehydrogenase. The incubation mixture (1.0 ml) contained: 50 mM triethanolamine-HCl, pH 7.5, $MgCl_2$ (4 mM), dithiothreitol (4 mM), NAD^+ (1.75 mM), $^{32}P_i$ (4.5 mM, 4.9×10^6 cpm/μmol), [^{14}C]glyceraldehyde 3-phosphate (1.46 mM, 6.1×10^7 cpm/μmol), glyceraldehyde-3-phosphate dehydrogenase (10 units), and phosphoglycerate kinase (20 μM). After incubation at room temperature for 2 min the enzyme was isolated from the reaction mixture on a Sephadex G-25 column (same conditions as in Figure 1).

Results and Discussion

Isolation of the Phosphoryl-Enzyme. Following the procedure of Walsh and Spector (1971), yeast phosphoglycerate kinase was incubated with [^{3}H,γ-^{32}P]ATP and the resulting ADP was rephosphorylated using a coupled enzyme system consisting of phosphoenolpyruvate and pyruvate kinase. After gel filtration (Figure 1) the protein fractions contained ^{32}P but no ^{3}H. The extent of phosphorylation of enzyme that could be achieved varied with different commercial enzyme preparations and was dependent upon the conditions of isolation. Isolated phosphoryl-enzyme samples contained from 0.01 to 0.5 mol of ^{32}P/mol of enzyme, and this proportion was independent of the enzymes' specific (catalytic) activity. The purified horse muscle enzyme (see Appendix) could not be phosphorylated to more than 1.5%. Maximal phosphorylation of any batch of yeast enzyme was achieved after 1 h. The phosphoryl-enzyme isolated by the above procedure was stable for several days at +4 °C and the rate of phosphate loss at 30 °C and pH 7.5 was found to be 0.00052 min^{-1} (Table II).

The phosphoryl-enzyme is chemically competent in that it is capable of the complete transfer of its phosphoryl group to either of the cosubstrates, ADP and 3-PGA. Both these transfers have also been demonstrated by previous workers (Walsh and Spector, 1971).

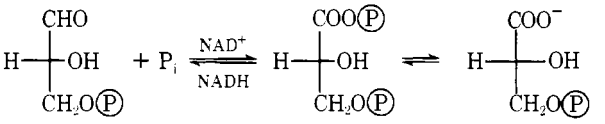
Partial Exchange Reactions. If the reaction catalyzed by phosphoglycerate kinase involves a discrete phosphoryl-enzyme, then the enzyme will catalyze an ATP-[^{3}H]ADP exchange in the absence of 3-PGA, and a BPGA-[^{14}C]3-PGA exchange in the absence of ADP, according to the partial reactions of Scheme I. It has previously been shown that the kinases from both rabbit muscle (Walsh and Spector, 1971) and from yeast (Roustan et al., 1973) do catalyze the ATP-ADP exchange, but the reactions are very slow and are stimulated more than 10^3-fold by 1 mM 3-PGA. We have also demonstrated this exchange for the horse muscle enzyme (Johnson et al., 1975).

Scheme I: Stepwise Phosphoryl Transfer Pathway for Phosphoglycerate Kinase.

$$\text{enzyme} + \text{ATP} \rightleftharpoons \text{enzyme} \sim \text{P} + \text{ADP}$$
$$\text{enzyme} \sim \text{P} + \text{3-PGA} \rightleftharpoons \text{enzyme} + \text{BPGA}$$

Since any contaminating kinase could catalyze the ATP-ADP exchange, we decided to investigate the BPGA-3-PGA partial reaction, which—for reasons of enzyme specificity—is much less likely to be an artifact arising from an enzyme contaminant. The instability of BPGA in aqueous solution ($t_{1/2}$, 27 min at pH 7.2, 38 °C: Negelein, 1974) was overcome by equilibrating BPGA with D-glyceraldehyde 3-phosphate and P_i in the presence of glyceraldehyde-3-phosphate dehydrogenase and NAD^+, as shown in Scheme II. If suitable initial concentrations of D-glyceraldehyde 3-phosphate, NAD^+, and P_i are chosen, the concentration of BPGA is small enough for the net turnover of D-glyceraldehyde 3-phosphate to 3-PGA (*via* BPGA) to be negligible. If sufficient coupling enzyme (glyceraldehyde-3-phosphate dehydrogenase) and cofactors are added to the system, then the BPGA-3-PGA exchange

Scheme II: System to Study the Partial Exchange Reaction of Phosphoglycerate Kinase.



glyceraldehyde phosphate dehydrogenase

phosphoglycerate kinase (in presence of [^{14}C]3-PGA but no ADP)

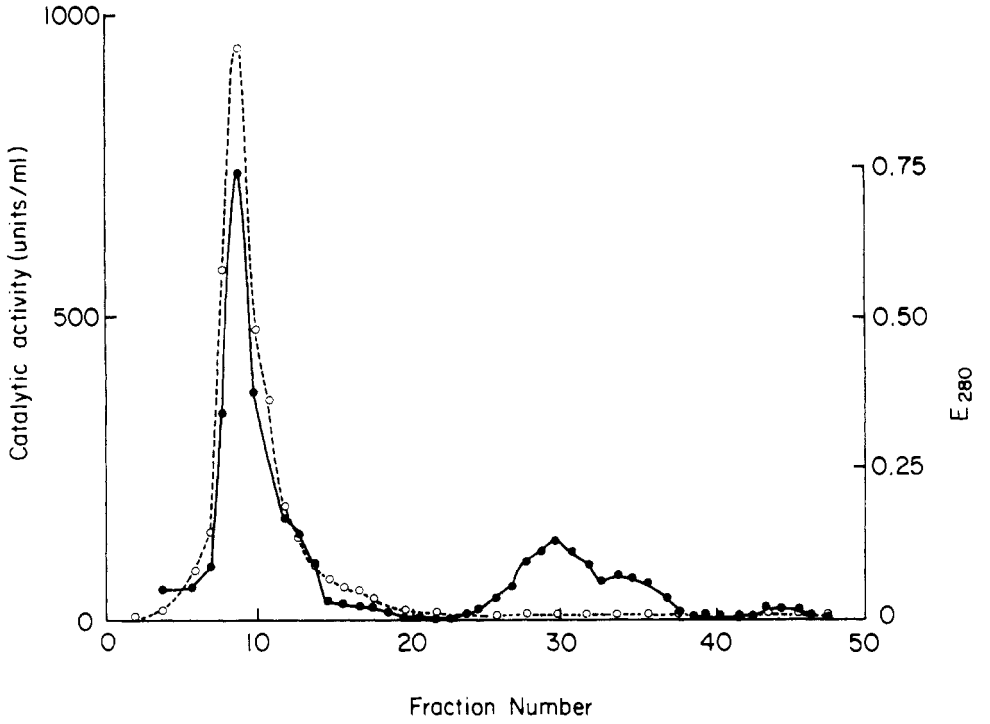


FIGURE 2: Isoelectric focusing of phosphoglycerate kinase. Yeast phosphoglycerate kinase (prepared by the method of Scopes, 1971) was dialyzed exhaustively into 5 mM triethanolamine-HCl, pH 7.5. Kinase (11.2 mg, 640 units/mg) was applied to a column (LKB:110 ml) and focused in a gradient of pH 5–8 at 22 °C. (Similar results were obtained at 4 °C.) After 48 h the column was eluted and fractions were assayed for phosphoglycerate kinase activity (O- - - -O), E_{280} (●—●), and ATP-ADP, and 3-PGA-BPGA exchange activities (data not shown).

becomes rate limiting. All the isotopic exchange reactions were carried out with the system initially at chemical equilibrium. This allows the experimental data to be analyzed in terms of a simple first-order reaction (see Methods).

Both the partial exchange reactions showed a magnesium dependence very similar to the overall reaction. The rate of each was proportional to the kinase concentration (from 0 to 30 μM), but was markedly slower than the overall catalyzed reaction, and was accelerated more than 10^3-fold by the cosubstrate at 1 mM.

In summary, it is possible to demonstrate the existence of each of the two partial reactions of Scheme I, though each of them is markedly accelerated by the addition of the second substrate. It is possible that Scheme I is an oversimplification, and that "substrate synergism" is responsible for the accelerations caused by the cosubstrates. Accordingly, cosubstrate analogues were sought that would be synergistic without being actual substrates. In the ATP-ADP exchange reaction, the following compounds were used as analogues of 3-phospho-D-glyceric acid: 3-phospho-L-glyceric acid, D-glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, phospho-D-serine, DL-α-glycerophosphate, phosphohydroxypyruvic acid, phosphoglycolic acid, phosphoglycolohydroxamic acid, 3-phospho-D-glycerylhydroxamic acid, and D-glyceric acid 3-sulfate. The instability to ester hydrolysis of the (carboxyl-) methyl ester of 3-phospho-D-glyceric acid prevented the use of this compound as a substrate analogue. At a concentration of 1 mM, *none* of the compounds tested, with the exception of 3-phospho-D-glycerylhydroxamic acid, had any significant effect (either stimulatory or inhibitory) on the rate of the ATP-ADP exchange catalyzed by either the horse or yeast enzymes. The hydroxamate at 1 mM concentration stimulated the exchange by ca. 50-fold, but it was subsequently shown that the acceleration was probably due to an extremely small 3-PGA contamination of ca. 0.3% (the hydroxamate was synthesized from 3-PGA either enzymatically or chemically). This level of contamination can readily account for the acceleration observed.

In the absence of positive results from the effects of substrate analogues on the rate of this partial exchange reaction and because of the irreproducible extent of phosphorylation of the enzyme it was decided to investigate more fully the possibility of contaminants either in substrates or in the enzymes.

It was proposed by Larsson-Raźniekiewicz and Schierbeck (1974) that the ATP-ADP exchange reaction was catalyzed not by phosphoglycerate kinase, but by contaminating adenylate kinase in the commercial yeast enzyme preparation used. However, in our preparation of yeast phosphoglycerate kinase (isolated according to Scopes, 1971) the content of adenylate kinase (<0.00003%, on an activity basis) was sufficient only to account for less than 1% of the observed ATP-ADP exchange rate. (For this experiment, rabbit muscle adenylate kinase was used, but since the turnover numbers for the yeast and rabbit enzymes are the same (Noda, 1973), this species difference cannot affect the conclusion.) Moreover, adenylate kinase does not, of course, catalyze the BPGA-3-PGA exchange. Adenylate kinase contamination cannot, therefore, explain the observed results.

Although our preparations of phosphoglycerate kinase from both horse muscle and from yeast were apparently homogeneous on polyacrylamide gel electrophoresis in sodium dodecyl sulfate, it was still possible that the slow exchange activities were due to catalysis by minor protein contaminants. The yeast enzyme was therefore subjected to isoelectric focusing. The isoelectric point of the enzyme was found to be 7.2 at both 4 and 20 °C (Figure 2). The following isoelectric points for the yeast enzyme have been quoted in the literature: 7.2 (Krietsch and Bücher, 1970); 7.02 (Stinson, 1974), and 7.61 (Brake and Weber, 1974). Figure 2 also shows that although a small amount of inactive protein is observed of p*I* 5.9, the major

protein peak, the overall catalytic activity, *and* the two partial exchange activities occur at the same p*I* value. Since after isoelectric focusing the activity of the enzyme had fallen to 70% of its original value yet the extent of phosphorylation was down to 10% of the original value, it seemed possible that isoelectric focusing had removed some low-molecular-weight contaminant that was causing the apparent phosphorylation of the enzyme. The results suggest (but cannot prove) that the two exchange activities are not due to contaminating enzymes. It had earlier been suggested that the phosphoryl-enzyme for phosphoglycerate kinase was in fact a phosphorylated protein impurity of low molecular weight which cochromatographed with the kinase on Sephadex G-25 (Larsson-Raźniekiewicz and Schierbeck, 1974). However, when the phosphoryl-enzyme was isolated on G-100 rather than G-25, in the expectation that such treatment would separate the kinase from such a notional phosphorylated impurity of low molecular weight, the kinase activity coincided exactly with the E_{280} and the ^{32}P radioactivity.

The above results do not rule out the possibility of contaminating cosubstrate, which would produce artifactual partial exchange activity. In our hands, a detectable increase in the rate of *either* of the partial exchange reactions is produced by added substrate at concentrations less even than that of the kinase itself.

The Real Nature of the "Phosphoryl-Enzyme." The equilibrium position for the phosphoglycerate kinase catalyzed reaction has been calculated as 3×10^3 in the direction of ATP production (Krietsch and Bücher, 1970). It should be possible, therefore, to form the phosphoryl-enzyme by incubation of the enzyme with BPGA, prepared in situ from D-[^{14}C]glyceraldehyde 3-phosphate, $^{32}P_i$, and glyceraldehyde-3-phosphate dehydrogenase. Using this approach to prepare the phosphoryl-enzyme, it was found that the horse and yeast enzymes could be phosphorylated rapidly and reproducibly to ca. 50% (Johnson et al., 1975). However, the enzyme bound an *equivalent* amount of ^{14}C and ^{32}P, indicating that the product was either the enzyme:BPGA complex, or the phosphoryl-enzyme with 3-PGA very tightly bound to it. There is, unfortunately, no suitable 3-PGA trap which does not include the use of ADP, so it was not possible to remove the 3-PGA from the incubation directly.

As a consequence of the variability of the isolation of the phosphoryl-enzyme from ATP, its kinetic incompetence, and the fact that a complex of enzyme with BPGA has a dissociation constant low enough for this complex to survive gel filtration, it seemed likely that the reported phosphoryl-enzyme was in fact an enzyme:BPGA complex. To test this, a solution of the "phosphoryl-enzyme" after gel filtration was boiled for 10 min to remove and hydrolyze all bound BPGA, and the precipitated enzyme was removed by centrifugation. The 3-PGA content of the solution was then determined. As can be seen from Table I, there is a close correlation between the achievable formation of "phosphoryl-enzyme", as measured by enzyme-bound ^{32}P, and the 3-PGA content of this species after hydrolysis. This is also true for "phosphoryl-enzyme" preparations in which extraneous 3-PGA is added to the incubation mixtures. It appears, therefore, that in the presence of ATP and an ADP trap, phosphoglycerate kinase scavenges the reaction mixture for 3-PGA, and the enzyme:BPGA complex is formed in amounts dependent upon levels of 3-PGA contamination of the solutions used.

To confirm this conclusion, the rates of hydrolysis of the "phosphoryl-enzyme" formed from [γ-^{32}P]ATP and of bona fide enzyme:BPGA prepared from D-[^{14}C]glyceraldehyde 3-phosphate and $^{32}P_i$ were determined at pH 7.5 and 2.7. As can be seen from Table II, the hydrolysis rates for both species are very similar at the two pH values. The hydrolytic half-life for BPGA when bound to the enzyme is more than an order of magnitude longer than that of free BPGA.

Since neutral hydroxylamine causes the facile release of P_i from the phosphoryl-enzyme, it has been suggested that the phosphoryl group is bound to the enzyme as an acyl phosphate (Walsh and Spector, 1971). However, although 0.15 M hydroxylamine causes the release of P_i, the phosphoglycerate kinase is *not* inactivated. The rate of loss of P_i from "phosphoryl-enzyme" is the same as from the enzyme:BPGA complex, and in both cases the kinase retains full catalytic activity. On treatment of the isolated "phosphoryl-enzyme" with 3 M hydroxylamine (the conditions used by Brevet et al., 1973)

TABLE I: Content of 3-Phosphoglycerate in Various Preparations of "E ~ P".

Enzyme	Ratio of Bound ^{32}P (as "E ~ P") to the 3-PGA Content of "E ~ P"[a]	% Phosphorylation[b]
Horse muscle[c]	—	<1.5
Yeast[d]	—	<1
Yeast,[e] lot 1	—	<1
Yeast,[e] lot 2	1.02	17.0
Yeast,[e] lot 3	0.95	12.7
Yeast,[e] lot 3	1.01	16.5
Yeast,[e,f] lot 3	0.96	42.4
Yeast,[e,g] lot 2	1.14	53.0

[a] Ratio of μmol of ^{32}P bound to the enzyme after gel filtration (after correction for the fall in specific radioactivity of the ATP during the incubation) to the μmol of 3-phospho-D-glycerate assayed after denaturation and hydrolysis. [b] Micromoles of bound $^{32}P/\mu$mol of enzyme (%). [c] Crystalline enzyme of specific activity 700 units/mg, prepared according to the method described in the Appendix. [d] Enzyme of sp. act. 740 units/mg, prepared according to Scopes (1971). [e] Different commercial preparations. [f] Incubation solution contained added 3-PGA in 1.5-fold molar excess over enzyme. [g] Incubation solution contained added 3-PGA in tenfold molar excess over enzyme.

TABLE II: Hydrolysis Rates of "E ~ P" and of E:BPGA (30 °C).[c]

	$k_{hydrolysis}$ at pH 7.5 (min^{-1})	$k_{hydrolysis}$ at pH 2.7 (min^{-1})
"E ~ P"[a]	0.00052	0.0089
E:BPGA[b]	0.00044	0.0081

[a] "E~P" was prepared by the method of Walsh and Spector (1971), from [γ-^{32}P]ATP and enzyme in the presence of an ADP trap. [b] Bona fide E:BPGA was prepared from $^{32}P_i$ and [^{14}C]glyceraldehyde 3-phosphate in the presence of catalytic amounts of glyceraldehyde-3-phosphate dehydrogenase. The ^{32}P:^{14}C ratio was 0.92:1.0. [c] "E ~ P" (0.98 μM; 3.08×10^8 cpm/μmol) prepared as described in Figure 1, E:BPGA (3.86 μM; 1.8×10^8 cpm/μmol) prepared from glyceraldehyde 3-phosphate, and P_i as described under Methods were incubated at 30 °C. At time intervals samples (10 μl) were taken and applied to thin-layer plates (Brinkmann MN polygram cel 300 PEI), which were developed in 1.0 M LiCl, pH 7.0, at 4 °C for 2.5 h. (R_f values: "E ~ P"/E:BPGA, 0.02; P_i, 0.45). The "E~P"/E:BPGA and P_i zones were excised and counted. The first-order rate constants for the hydrolysis rates were calculated from plots of log [(total cpm − cpm in P_i)/total cpm] vs. time.

there is no loss of catalytic activity over a period of 3 h, even though under these conditions the phosphate group is released rapidly. If the "phosphoryl-enzyme" were, as has been proposed (Brevet et al., 1973), an acyl phosphate covalently linked to the γ-carboxyl group of a glutamic acid residue, an inactive enzyme hydroxamate would be expected. The present result is nicely explained in terms of an enzyme:BPGA complex: treatment with hydroxylamine would cause the facile release of P_i (BPGA contains the acyl-phosphate linkage) but would not inactivate the enzyme. Brevet et al. (1973) have claimed that the hydroxylaminolysis of their "phosphoryl-enzyme," followed by a Lossen rearrangement, leads to the formation of some 2,4-diaminobutyric acid derived from a glutamyl residue. This finding conflicts with the fact that, under the conditions of hydroxylaminolysis used by Brevet et al. (1973), the resulting dephospho-enzyme has full catalytic activity. Brevet et al. (1973) did not report the catalytic activity of their enzyme preparation after hydroxylamine treatment. Finally, the observation of a pH dependence for "phosphoryl-enzyme" hydrolysis characteristic of an acyl phosphate (Walsh and Spector, 1971) is, of course, entirely consistent with the species being a complex between kinase and BPGA.

In the above discussion we have suggested that the "phosphoryl-enzyme" is actually an enzyme:BPGA complex. However, our results do not distinguish between this complex and a phosphoryl-enzyme containing tightly bound 3-PGA. If the species is in reality E ~ P:PGA then it would have to be in rapid equilibrium with enzyme:BPGA to accommodate the hydroxylaminolysis results.

The mechanistic pathways of phosphotransferases are routinely divided into one of two classes, involving either a single or a double displacement (Morrison and Heyde, 1972; Spector, 1973). In the double displacement mechanism, the enzyme reacts with the first substrate alone to form a product that is released before the cosubstrate binds. This implies that (a) it may be possible to isolate a covalent intermediate (which should be both chemically and kinetically competent), and (b) partial isotopic exchange reactions of appropriate rates may be observed. In the single displacement mechanism, group transfer occurs directly between substrate molecules within a ternary complex, and in this case no partial exchange reactions should be observed in the absence of cosubstrate.

In the literature there are numerous examples of enzymes that catalyze partial exchange reactions which are stimulated by the presence of the cosubstrate. The slowness of the exchange reactions has been variously explained away by (i) the presence of contaminating enzymes, (ii) the presence of contaminating cosubstrate, (iii) the double displacement mechanism as a secondary and unimportant route, and (iv) the concept of substrate synergism.

In the case of phosphoglycerate kinase we have shown that the reported phosphoryl-enzyme is in fact a tight complex between the kinase and BPGA, possibly in equilibrium with E ~ P:PGA. The evidence in favor of a double displacement pathway, deriving from the isolation of a phosphoryl-enzyme and from the observation of partial exchange reactions, is therefore in doubt.

In contrast, steady-state kinetic analyses of the phosphoglycerate kinase catalyzed reaction have been interpreted as favoring a sequential mechanism with random addition of substrates (Larsson-Raźniekiewicz and Arvidsson, 1971; Janson and Cleland, 1974; Lee and O'Sullivan, 1975). It is important to realize, however, that the steady-state kinetic approach does not provide any evidence about the actual chemical nature of the phosphoryl transfer. In the case of phosphoglycerate kinase, the only valid conclusion to be drawn from these studies is that ternary complexes involving enzyme:ATP:3-PGA and enzyme:ADP:BPGA *may* be part of the catalytic pathway. It is quite possible that the phosphoryl transfer within the ternary complex is either direct (between substrates) or indirect (via a phosphoryl-enzyme). There is as yet no definitive method to differentiate between these two possibilities. It is apparent from the work described in this paper and elsewhere (cf. Switzer and Simcox, 1974) that one has to treat the existence of slow partial exchange reactions with great caution. No mechanistic interpretations should be placed on such results unless it can be shown that they are not caused by very minor contamination of substrates, or of enzymes. It is also apparent that there is no real distinction between a "substrate-synergized" ping-pong mechanism and phosphoryl transfer *via* the enzyme within a ternary complex. We are not suggesting that "substrate synergism" is not a real phenomenon, but that the observation of genuine—if slow—partial exchange reactions is indicative of intermolecular phosphoryl transfer within a ternary complex, and is more usefully classed with the normal sequential mechanism, rather than being viewed as a modification of the ping-pong pathway.

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Appendix

ABSTRACT: Phosphoglycerate kinase has been isolated in crystalline form from horse muscle. A convenient isolation procedure is described that yields homogeneous enzyme of specific activity 700 units/mg (30 °C). The enzyme is monomeric, and has a molecular weight 47 000. Of the eight cysteine residues in the protein, two react rapidly with Nbs_2[1] with the concomitant loss of the catalytic activity.

Since the isolation of phosphoglycerate kinase from yeast (Bücher, 1955) there have been several reports of purification methods yielding enzyme approaching molecular homogeneity, from rabbit muscle (Beisenherz et al., 1953; Czok and Bücher, 1960; Rao and Oesper, 1961; Avramov and Repin, 1965; and Scopes, 1969) and from chicken muscle (Gosselin-Rey, 1965). Crystalline material has been isolated from human erythrocytes (Hashimoto and Yoshikawa, 1962), and from yeast and rabbit muscle (Krietsch and Bücher, 1970). Cystallographic work on phosphoglycerate kinase from horse muscle by Blake et al. (1972) and Blake and Evans (1974) has prompted mechanistic interest in the enzyme, and we report here a simplified isolation procedure and some properties of the crystalline material from this source.

Materials and Methods

Materials. Phosphocellulose (P11) was obtained from W. & R. Balston Ltd. (England). Fresh horse meat was obtained from Wessex Biochemicals Ltd. (England) and was stored at −20 °C. All other reagents were purchased from sources as described in the main paper.

Methods. Phosphoglycerate kinase was assayed by coupling the formation of 1,3-bisphosphoglycerate with the glyceraldehyde-3-phosphate dehydrogenase reaction (Bücher, 1955). The assay mixture contained 3-phosphoglycerate (10 mM), ATP (4 mM), $MgCl_2$ (6 mM), EDTA (200 μM), NADH (200 μM), in 50 mM imidazole-HCl buffer, pH 7.2. The solution also contained glyceraldehyde-3-phosphate dehydrogenase (ca. 30 μg/ml), and the reaction was initiated by the addition of the kinase sample (100 μl, into 2.9 ml of assay mixture). The absorbance at 340 nm was monitored in a Unicam SP 1800 recording spectrophotometer, at 30 °C. A molar extinction coefficient for NADH of 6220 M^{-1} cm^{-1} was assumed (Horecker and Kornberg, 1948). A unit of activity is defined as amount of enzyme required to consume 1 μmol of 3-phosphoglycerate/min under these conditions.

Myokinase activity was assayed by the method of Chiu et al. (1967).

Protein was determined by measurement of E_{280}.

Polyacrylamide gel electrophoresis was done as described by Davis (1964), and in the presence of sodium dodecyl sulfate, as described by Shapiro et al. (1967).

Amino acid analysis was done on a Jeol JLC 5AH instrument. Protein samples were hydrolyzed in vacuo in 6 N HCl containing phenol (0.5% w/v) for 22, 48, or 96 h at 110 °C. The tryptophan content was determined by the method of Previero et al. (1967). Cysteine was determined by the method of Hirs (1967) as cysteic acid after performic acid oxidation of the protein followed by the usual 6 N HCl hydrolysis for 22 h.

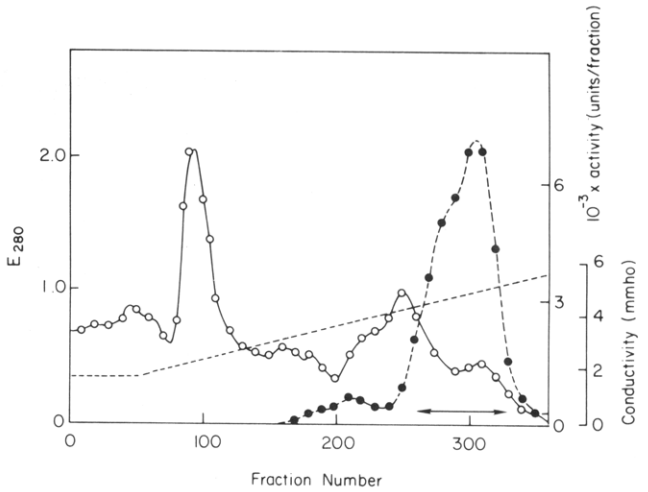

FIGURE 1A: Elution profile from phosphocellulose column. For details, see the text. Fractions 260–330 were pooled for step V. E_{280} (—○—); catalytic activity (- -●- -); conductivity (·····).

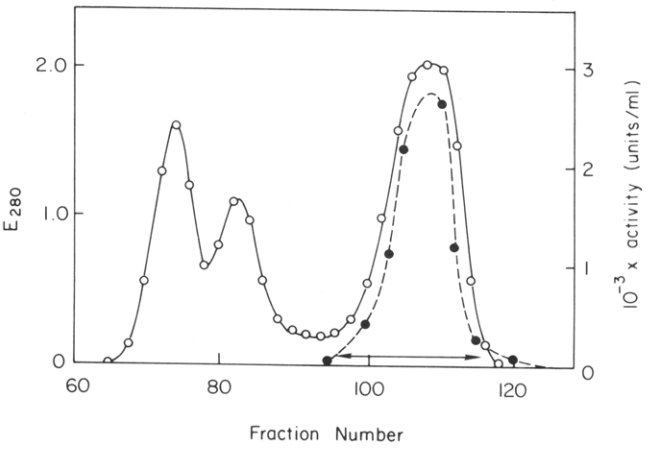

FIGURE 2A: Elution profile from Sephadex G-100 column. For details, see the text. Fractions 96–116 were pooled. E_{280} (—○—); catalytic activity (- -●- -).

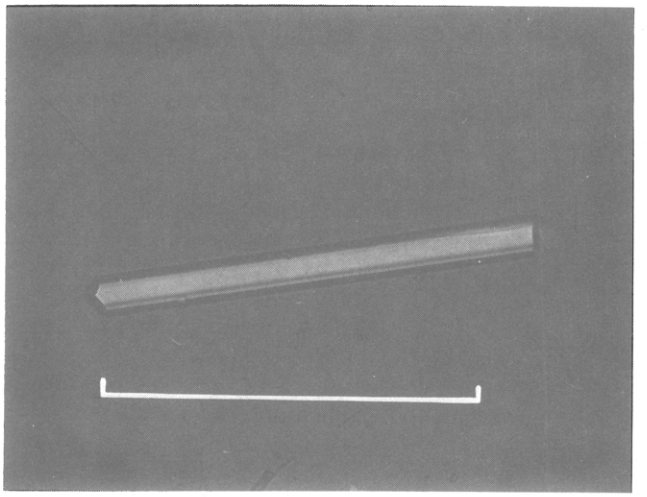

FIGURE 3A: Crystalline phosphoglycerate kinase from horse muscle. The scale bar is 1 mm.

TABLE IA: Purification of Phosphoglycerate Kinase.[a]

Fraction	Vol. (ml)	Total Protein (mg)	10^{-3} × Total Act. (units)	Sp Act. (units/mg)	Purification	Yield (%)
I, extract	6000	240 000	660	3.4	1	100
II, ammonium sulfate fraction (2.5–3.3 M)	500	54 500	615	14.0	4.1	93
III, DEAE-cellulose treatment	1430	20 400	550	33.4	9.8	89
IV, phosphocellulose column	1820	1400	320	282	84	48
V, ammonium sulfate concentration	25	645	202	390	117	31
VI, G-100 column	120	358	202	700	210	31

[a] For details, see the text.

TABLE IIA: Amino Acid Analysis of Phosphoglycerate Kinase from Horse Muscle.[a]

Amino Acid	22-h Hydrolysate	48-h Hydrolysate	96-h Hydrolysate	Average or Extrapolated Value
Lys	40.6	40.4	40.3	40.5
His	5.24	5.34	5.38	5.31
Arg	10.76	10.70	10.38	10.6
Asp	47.22	47.05	46.24	46.8
Thr	17.32	16.03	15.01	18.2[b]
Ser	22.60	18.24	14.70	25.6[b]
Glu	34.70	34.60	34.80	34.7
Pro	17.78	18.22	19.17	18.4
Gly	43.31	43.10	42.13	43.1
Ala	44.10	43.90	44.31	44.1
Cys	7.71			7.71[c]
Val	40.80	41.65	42.06	42.1[d]
Met	13.00	13.03	12.90	13.0
Ile	18.31	18.83	18.98	19.0[d]
Leu	40.60	40.12	40.80	40.5
Tyr	4.56	4.41	4.67	4.54
Phe	16.40	16.10	16.52	16.4
Trp				3.9[e]

[a] Each result is the average of two analyses. [b] Value from extrapolation to zero time. [c] Determined after performic acid oxidation of the

The optical factor was determined by the dry weight method, as practiced by McVittie et al. (1972).

For polarographic work, a Radiometer instrument type PO-4 recording polarograph was used.

Results and Discussion

Isolation of the Enzyme. All operations were done at 0–4 °C unless otherwise stated.

I. Extraction. Frozen horse meat (3 kg) was thawed, homogenized in a Waring blender, and stirred for 45 min with 20 mM Tris-HCl buffer (6 l.), pH 7, containing EDTA (2 mM), 2-mercaptoethanol (10 mM), and glycerol (2% v/v). After centrifugation at 13 000*g* for 15 min, the supernatant (6 l.) was taken to pH 5.4 with 5 M acetic acid (fraction I).

II. Ammonium Sulfate Precipitation. This was done at room temperature. To fraction I, solid ammonium sulfate was added slowly to 2.0 M (310 g/l.). This was left for 1 h, and a further 90 g/l. of ammonium sulfate was added to bring the concentration to 2.5 M. Centrifugation at 13 000*g* for 30 min gave a dark supernatant, to which a further 165 g/l. of ammonium sulfate was added to bring the concentration to 3.3 M. This solution was left at 4 °C overnight, then centrifuged at 4 °C as above. The resulting pellet was suspended in Tris-cacodylate buffer (500 ml) [containing Tris (10 mM), EDTA (200 μM), 2-mercaptoethanol (10 mM), adjusted to pH 8.0 with 1.0 M cacodylic acid] to give a very dark-colored fraction II.

III. DEAE-Cellulose Treatment. Fraction II was dialyzed exhaustively against the Tris-cacodylate buffer, and then added to DE-52 (1 l. equilibrated against the same buffer). After standing for 1 h, the cellulose was removed by filtration, and washed with the same buffer (ca. 1 l.). The combined filtrate and washings, which were now pale yellow, were dialyzed exhaustively against 50 mM sodium phosphate buffer, pH 6.6, containing EDTA (200 μM) and 2-mercaptoethanol (10 mM) (fraction III). Light precipitation was removed at this stage by centrifugation.

IV. Phosphocellulose Chromatography. Fraction III was applied to a column (5 × 56 cm) of phosphocellulose equilibrated with 50 mM sodium phosphate buffer, pH 6.6, containing EDTA (200 μM) and 2-mercaptoethanol (10 mM). The column was then washed with buffer (1 l.) and the enzyme eluted with a linear gradient (10 l.) of sodium phosphate buffer (50–200 mM). The flow rate was 80 ml/h. Fractions of 27 ml were collected. The elution profile from this column is shown in Figure 1A. Fractions of more than twice the specific activity of fraction III were pooled and concentrated by ammonium sulfate precipitation (fraction IV).

V. Concentration. The proteins in fraction IV were precipitated by the addition of ammonium sulfate to 3.3 M. The pellet obtained after centrifugation at 23 000*g* for 40 min was suspended in 20 mM Tris-HCl buffer, pH 8.0, containing KCl (150 mM), EDTA (200 μM), and 2-mercaptoethanol (10 mM), and dialyzed exhaustively against the same buffer.

VI. Sephadex G-100 Chromatography. Fraction V was applied to a column (5 × 85 cm) of Sephadex G-100 (medium), equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing KCl)150 mM), EDTA (200 μM), and 2-mercaptoethanol (10 mM), and eluted with the same buffer at 40 ml/h. Fractions of 8 ml were collected, and those of more than twice the specific activity of fraction V were pooled. The elution profile from this column is shown in Figure 2A. The enzyme was precipitated by the addition of ammonium sulfate to 3.3 M, and stored as a suspension at 0 °C. For crystallization, the protein was dialyzed against 50 mM Tris-HCl buffer pH 7.4, containing EDTA (2 mM) and 2-mercaptoethanol (10 mM). Precipitation with solid ammonium sulfate at 0 °C was followed by successive extraction according to the method of Jakoby (1968). Large crystals grew over a few days (see Figure 3A). A summary of the purification is given in Table IA.

Properties of the Enzyme

Molecular Weight. As has been noted by other workers (Walsh and Spector, 1971), phosphoglycerate kinase does not readily penetrate the usual 7.5% polyacrylamide gel on electrophoresis. In the presence of sodium dodecyl sulfate, however, a single sharp band is obtained, even at high loadings of protein. The molecular weight of the kinase determined by this method is 47 400 ± 2000. In the ultracentrifuge, the enzyme sediments as a single component. No evidence for aggregation is apparent. Assuming a value of $\bar{v}$ of 0.75 the molecular weight under equilibrium conditions using the method of Chervenka (1966) was found to be 47 000. These values are close to that of 48 000 reported for the enzyme earlier (Blake et al., 1972) and to the values of 47 100 (from gel filtration) and 46 900 (from ultracentrifugation) reported for the rabbit muscle enzyme by Krietsch and Bücher (1970). From the close agreement between the results from ultracentrifugation of the native enzyme and from gel electrophoresis under reducing and dissociating conditions, it appears that the enzyme is monomeric.

Optical Factor. The optical factor ($E_{280}^{0.1\%}$) of the kinase, as determined by replicate dry weight measurements, was found to be 0.684. Similarly low values have been reported for the rabbit muscle enzyme, of 0.69 (Krietsch and Bücher, 1970) and 0.57 (Scopes, 1969) for the chicken muscle enzyme of 0.57 (Gosselin-Rey, 1965), and for the yeast enzyme of 0.49 (Krietsch and Bücher, 1970).

Amino Acid Composition. The amino acid composition of the horse muscle enzyme (Table IIA) is, with the exception of the basic amino acids, very close to that reported for the rabbit enzyme (Krietsch and Bücher, 1970).

Activity and Stability. The specific activity of our preparation is 700 units/mg (30 °C), which agrees with the value of 600 units/mg (25 °C) found by Krietsch and Bücher (1970), though not with Scopes' value (1969) of 975 units/mg (30 °C) for the rabbit muscle enzyme.

Phosphoglycerate kinase from horse muscle is very sensitive to air oxidation and to heavy metals, and should be stored in the presence of thiols and EDTA to maintain maximal activity. Kinase that has lost activity can be reactivated to the full specific activity by dialysis at 4 °C against a Tris-HCl buffer (50 mM, pH 7.4) containing EDTA (2 mM) and fresh 2-mercaptoethanol (10 mM). The myokinase and ATPase contents of the preparation are less than 0.002% on an activity basis. It is important that such activities be minimal if any studies on the interaction of the enzyme with ATP (see the main paper) are contemplated.

Thiol Groups. The sensitivity of the enzyme to oxidation invited some investigation of the number and role of cysteine residues in the protein. From the amino acid analysis of performic acid oxidized enzyme, 7–8 residues of cysteic acid were found. The cysteine content of the enzyme was determined by three other methods. Amperometric titration of the enzyme (ca. 20 μM) with *p*-chloromercuribenzoate in the presence of sodium dodecyl sulfate (1% w/v) was performed according to the method of Cecil and Snow (1962), and indicated 8.1 free thiol groups. Spectrophotometric titration with *p*-chloromercuribenzoate under the same conditions by the method of Boyer (1954), gave a value of 8.7 thiol groups. Titration of the enzyme with Nbs_2 (Ellman, 1959) in the presence of sodium dodecyl sulfate (0.2% w/v) gave 8.1 free thiol groups. In the absence of denaturing agent, reaction of the enzyme with Nbs_2 results in the rapid titration of 2.3 thiol groups within 30 s with concomitant loss of enzyme activity, followed by the slower reaction of the other groups. From these results, the enzyme apparently contains two fast- and six slow-reacting thiol groups.

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